Imaging the accumulated intracellular microalgal lipids as a response to temperature stress

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Abstract Over the last few decades, many scientists considered microalgae as promising actors for future biofuels because of the high lipid productivity inside their cells. Moreover, much attention has been paid to algal lipids as they can be used in biodiesel production. In this study, we optimized the different suitable conditions such as incubation time, incubation temperature, Dimethylsulfoxide and Nile red concentrations of the lipophilic fluorescence dye Nile red as an excellent and fast vital stain to detect and quantify intracellular lipids. This was achieved using the green alga *Nannochloropsis salina*. In addition, investigating the accumulation of lipid vesicles inside different isolated microalgal species as a response to temperature stress. Furthermore, the confocal laser scanning microscopy (LS510) for imaging and measuring the size and volume of the accumulated lipid vesicles was used.

Keywords Microalgae · Nile red (NR) · Neutral lipids · Fluorescence dye · Scanning microscopy

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Introduction

Microalgae have received global attention as a promising sustainable feedstock for biofuel production as they use solar energy and convert it to lipids in the form of Triacylglycerols (TAGs) through the process of photosynthesis (Gusbeth et al. 2016; Ota et al. 2016; Zhu et al. 2016; Yu et al. 2011). Compared to other feedstocks, microalgae exhibit many advantages such as high photosynthetic efficiency, high growth rate, short doubling time and high lipid productivity (Meng et al. 2009; Widjaja et al. 2009). Moreover, they can grow in non drinking water and on non arable lands in addition high CO₂ mitigation efficiency. Later, TAGs can be extracted and chemically transesterified to produce Fatty Acid Methyl Ester (biodiesel) which is considered a bottleneck in the biofuels industry.

Biodiesel is biodegradable, renewable, non-toxic, and an environmentally friendly source of energy; it does not pollute to the environment as normal diesel does as there is no emission of sulfur compounds when using biodiesel (Abou-Shanab et al. 2011; Collet et al. 2014; Knothe 2011; Wang et al. 2016; Wong and Franz 2013; Wahlen et al. 2011; Yao et al. 2012).

Lipids produced by microalgae can be grouped into two main categories; storage neutral lipids or non-polar lipids and structural phospholipids or polar lipids (Harwati et al. 2012; Velmurugan et al. 2013). Neutral lipids which are produced and accumulated during the stationary growth phase are mainly in the form of TAGs which represent a storage form of carbon and energy in the cell and can be used as an energy source (Cho et al. 2015). Moreover, microalgal TAGs are generally synthesized in the light, stored in cytosolic lipid bodies, and then reutilized for polar lipid synthesis in the dark. The polar lipids or phospholipids which are produced during the growth phase are

located in cell membranes and chloroplasts; they are important structural components of the cell membranes and act as a selective permeable barrier for cells and organelles, these lipids maintain the specific membrane functions providing the matrix for a wide variety of metabolic processes (Sharma et al. 2012; Wong and Franz 2013).

The synthesis of lipids in oleaginous unicellular microalgae takes place under optimal conditions (Gong et al. 2013; Goncalves et al. 2016). TAGs formation in eukaryotes takes place in specialized organelles such as chloroplasts. However, in prokaryotes it takes place in the cytoplasm (Sakthivel et al. 2011).

All TAGs are synthesized through a complex pathway and by a single set of enzymes in the chloroplast. Initiation process of Acetyl-CoA formation takes place by the enzyme Acetyl CoA carboxylase (ACoAase) which is considered the most important and crucial step during lipid biosynthesis (Goncalves et al. 2016; Minhas et al. 2016). The synthesis of fatty acids starts with glycolysis derived pyruvate during the respiration process (Hu et al. 2008; Sharma et al. 2012).

In glycolysis, pyruvate kinase catalyzes the irreversible synthesis of pyruvate which is then converted into Acetyl CoA; the major compound used to produce fatty acids (Song et al. 2008; Sakthivel et al. 2011).

Micro-algal oil composition is similar to that of vegetable oils; however, other literature states that micro-algal oils are richer in polyunsaturated fatty acids with four or more double bonds (Chisti 2007). It can vary widely depending on the strain and culture conditions. Typically, oil levels of 1–26% (dry weight basis) are quite common (Dunstan et al. 1993). The composition and concentration of lipids in microalgae can be influenced by exposure to certain environmental conditions as variations in temperature, nutrients, salinity, pH, photoperiod, light intensity and light quality (Chen et al. 2011; Fabiano et al. 2006; Liu et al. 2008; Romano et al. 2000; Yamaberi et al. 1998).

Under unfavorable environmental or stress conditions, many microalgae alter their metabolic pathways towards the formation and accumulation of high amounts of neutral lipids from 20 to 50% DCW (dry cell weight), mainly in the form of TAGs in addition to other compounds such as carbohydrates and secondary metabolites, enabling microalgae to endure these adverse conditions (Meng et al. 2009; Sharma et al. 2012). The neutral lipids are mostly triglycerides that serve primarily as a storage form of carbon and energy, they can account for as much as 80% of the total lipid content in the cell (Song et al. 2008; Teo et al. 2014).

The temperature is one of the most important factors that greatly affect the rate of cell growth, lipid productivity and lipid composition. However, it is species dependent as many studies have shown that microalgal growth rate, as

well as lipid production increases when the temperature increases until the optimum level, which in turn differs from one species to another, but generally ranges between 20 and 27 °C (Zhu et al. 2016). Therefore, it is very important to select promising strains that show a remarkable increase in lipid productivity under exposure to high temperature (Minhas et al. 2016; Wang et al. 2016).

Different established screening methods were described for lipid quantification including solvent extraction, gravimetric determination, gas liquid chromatography (GLC) and high pressure liquid chromatography (HPLC). All of these methods are complicated, time consuming, and require extensive, sophisticated laboratory work (as they include pretreatment, preparation, extraction, purification, concentration and quantification of lipids) (Elsei et al. 2007; Park et al. 2013). Furthermore, a proper amount of algal biomass is required for extraction (Gusbeth et al. 2016). This is why many researchers have tried to develop fast in situ quantification methods such as lipo-fluorescent dyes like Nile red (9-diethylamino-5-benzo[α] phenoxazinone) and BODIPY (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene). These methods are more efficient, cheaper and easier to use compared to all of the previously mentioned techniques. Additionally, they are significantly time saving (Govender et al. 2012; Ren et al. 2013; Mishra et al. 2014; Cabanelas et al. 2015; Natunen et al. 2015).

NR offers several characteristics such as photo-stability, fluorescence in organic solvents and hydrophobic environments and allows us to distinguish between neutral lipids and polar lipids; furthermore, the fluorescence of NR was found to be linearly correlated with the amount of TAGs contained in microalgal cells (Kou et al. 2013; Rumin et al. 2015).

Materials and methods

Optimization of Nile red assay

The Nile red assay was repeated several times using model alga *Nannochloropsis salina* with cell numbers of 7×10^8 /ml, an optical density of 1 (OD₇₀₀) and using the recommended results presented by Chen et al. 2009. Therefore, different concentrations of DMSO (Dimethylsulfoxide 99.5%, Carl Roth GmbH + Co.KG, Germany) ranging between 5 and 30% v/v (final concentration/ml) were added. The stock solution of NR stain were prepared by adding 10 mg NR powder to 10 ml Acetone ($\geq 99.9\%$ HPLC grade, Carl Roth GmbH + Co.KG, Germany) (Doan and Obbard 2012).

The used NR final concentration ranged from 0.5 to $3 \mu\text{g ml}^{-1}$ was then vortexed for 30 s. They were then

incubated in dark conditions with different time intervals ranging from 10 to 60 min and an incubation temperature ranging between 10 and 70 °C. A HITACHI Fluorescence Spectrophotometer F-2500 (Hitachi, Germany) was used with excitation and emission at 530 and 580 nm, respectively. Data analysis was performed using FL Winlab software (scan speed 60 nm/min, excitation slit 5 nm, emission slit 20 nm and PMT voltage 700 V). All concentrations were measured in triplicates.

Standard lipid curve

For creating a standard lipid curve, Triolein (Glyceryl trioleate 65%, Sigma/Aldrich, USA) was used as a referenced lipid substance. 0.35 g of Triolein was added to 49.95 ml isopropanol (2-Propanol $\geq 99.8\%$, Carl Roth GmbH + Co.KG, Germany) then serial dilutions and calculations were performed to prepare different concentrations in triplicate starting from 0.001 to 0.01 g/l. The fluorescence intensity (FI) for each concentration was measured using the recommended results of our conducted experiment of Nile red assay (Fig. 1).

Isolation and cultivation of the tested species

The tested 12 microalgae species were obtained from microalgae culture collection at Hochschule Bremen, Germany. Originally, they were isolated from soil and

water samples of marine habitat (Red Sea). The samples were brought to the lab and the isolation process started immediately after the sample collection is completed because some species might be very sensitive and die when moved from their natural environment. Agar plate method with f/2 media was used to get unialgal colonies then each single colony was picked and either streaked again on agar plate or inoculated into liquid medium of f/2. This method repeated several times to finally get a pure unialgal culture. The molecular identification and phylogenetic analysis of these strains were done and we got these strains name from the results of BLAST analysis on NCPI data base.

Preparation of algal cells for lipid vesicles visualization

The cells of different isolated microalgae species were grown on f/2 media at a temperature of 40 °C in 12 h light and 30 °C in 12 h dark cycles, respectively, with light intensity of $105 \pm 2 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. The flasks were rotated at 150 rpm on an orbital shaker for 14 days. The cells were harvested using centrifugation for 6 min at 4000 rpm and cells were concentrated up to 1 ml. Nile red assay with recommended results was then applied to the concentrated cells at 20% DMSO as a final concentration in the reaction tube, adding 2 $\mu\text{g/ml}$ Nile red and incubated in the dark for 20 min at room temperature. They were then examined under laser microscope.

Fig. 1 Effect of different parameters on Nile red assay **a** NR concentration $\mu\text{g ml}^{-1}$ (w/v), **b** DMSO concentration % (v/v), **c** Incubation time (min) and **d** Incubation temperature (°C) on the fluorescence intensity (a.u.) of the green alga *N. salina* as a model organism. The excitation and emission wavelengths used were 530 and 580 nm, respectively, the OD_{700} of the algal suspension was 1. All the tested samples expressed as mean values with standard deviations of three replicates

Confocal laser scanning microscopy (CLSM)

Confocal imaging (1.0 Airy units) was performed with a laser scanning microscope LSM510 (Carl Zeiss, Germany) and a Plan-Apochromat100x/1.25 NA oil objective lens (Carl Zeiss). For imaging, an aliquot of cells was placed between two microscopy cover slips. Fluorescence of Nile red was analyzed using an Argon laser with 488 nm excitation wavelength; emission was collected using a 530–600 nm band-pass filter. Chlorophyll fluorescence was analyzed using HeNe laser with 633 nm excitation wavelength; emission was collected using a 650 nm long-pass filter. Images were merged and pseudo-colored in Carl Zeiss confocal software ZEN 2012 (blue edition). All microscopy experiments were performed in triplicate with different preparations of algal species. In each experiment, at least ten cells were analyzed to get sustained statistics of vesicle staining.

Results

Optimization of Nile Red assay

Nile red concentration

First, we tested the fluorescence intensity (FI) of the green alga *N. salina* without NR as a negative control. The average FI was comparatively small at 464 a.u. (Fig. 1). With a concentration of NR less than $2 \mu\text{g ml}^{-1}$ it is clear that it is not sufficient to stain all the neutral lipids inside the cells. By adding a different concentration of NR followed by fluorescence measurements at excitation and emission wavelengths of 530 and 580 nm, respectively, the FI increased at a concentration of $2 \mu\text{g ml}^{-1}$ (optimal concentration). The fluorescence intensity decreased after adding a more concentrated NR.

DMSO concentration

DMSO has proven to be more advantageous than any other organic solvent to improve NR stain effectiveness (Shrivastava and Gupta 2011). However, the optimal DMSO concentration should be known for correct measurements (Fig. 1). From the obtained results, it is clear that there was no remarkable difference between different DMSO concentrations. 20% DMSO displayed the highest fluorescence intensity with a low standard deviation of 10 a.u. By increasing the DMSO concentration the FI decreased.

Effect of incubation time and temperature

The Fluorescence intensity changed slightly with different incubation times. Different time intervals were tested,

starting from measuring the FI immediately after adding NR until 60 min. However, there was no significant difference between different time intervals. Therefore, it is recommended to take measurements at 20 min after adding NR (Fig. 1).

From the incubation temperature, it was clear that, there was no big difference between the incubation temperatures that were tested as can be seen with the low standard deviation (SD). This is why it is recommended that 20 °C is a suitable incubation temperature (Fig. 1).

Standard lipid curve

The FI of different concentrations of Triolein, 65% of a standard lipid compound (Sigma/Aldrich), ranged between 1×10^{-3} and 1×10^{-2} g/l and were measured following the recommended NR assay results to create the standard lipid curve. This was used later to calculate the lipid content during further experiments of temperature stress with low limit of detection (LOD) of 142 a.u. and low limit of quantification (LOQ) of 152 a.u. which were measured using the following equations:

$\text{LOD} = \mu + 3 \text{ SD}$ and $\text{LOQ} = \mu + 10 \text{ SD}$ where μ is the mean value of the blank and SD is the standard deviation of the blank (Morowvat et al. 2010).

Discussion

Nile red is a lipophilic fluorescent dye that fluoresces at a defined wavelength (Bertozzini et al. 2011); it has been proposed to determine the neutral lipid content inside microalgal cells (Rumin et al. 2015). Several trials that were performed to improve the efficiency of NR stain (Chen et al. 2009) revealed that adding DMSO at a concentration of 25% (v/v) in the staining solution increases the efficiency of Nile red to maximum fluorescence intensity. The measured fluorescence of the NR assay could be changed daily so it is important to take the time factor into consideration during measurements (Doan and Obbard 2012). Staining of algal cells growing at 40 °C for 10 min yielded optimal neutral lipid fluorescence. The concentration of NR 0.5 mg/ml was found to be the optimal concentration at cell densities ranging from 5×10^4 to 4×10^5 cells ml^{-1} (Chen et al. 2009).

Nile red is hydrophobic in nature as it can only be dissolved in organic solvents (Doan and Obbard 2012; Pick and Rachutin-Zalagin 2012). Many previous studies have shown, adding organic solvents like acetone, ethylene glycol or glycerol could improve the efficiency of NR staining assays. However, amongst the different solvents used, DMSO showed the highest efficiency for improving the penetration of NR through the rigid cell wall and other

cytoplasmic membranes inside living cells that we tested. DMSO improved the ability to stain the accumulated neutral lipids (Chen et al. 2009).

Based on previous investigations, the used excitation and emission wavelengths during this study were 530 and 580 nm, respectively (Chen et al. 2009). $2 \mu\text{g ml}^{-1}$ was found to be the optimal concentration during the measurement of fluorescence in the model green alga *N. salina*. On the other hand, NR stain was found to be slightly influenced by different incubation times and incubation temperatures but the fluorescence intensity at room temperature (20 °C) exhibited the highest value so it was considered as optimum temperature, which is consistent with the results obtained by Shrivastava and Gupta 2011 with *Nannochloropsis* sp. An alternative study was performed on green alga *Chlorella vulgaris* that stated, a staining time of 30 min and a temperature of 40 °C were the optimal conditions for the NR assay (Chen et al. 2009).

We found that 20% DMSO was optimal during fluorescence measurements; however, another study was conducted by (Doan and Obbard 2012) concluded that a concentration of 5% DMSO (v/v) with $1\text{--}2 \mu\text{g ml}^{-1}$ as a final concentration is optimal for measurements in *Chlamydomonas reinhardtii*. Although, there are different published results used the alga *Nannochloropsis* sp., showed 16.5% DMSO with an incubation time of 5 min with $0.7 \mu\text{g ml}^{-1}$ NR as a final concentration are the optimal conditions for NR assay (Shrivastava and Gupta 2011). From the previous discussion, it can be concluded that obtaining fluorescence data from the NR assay could be affected by a number of factors, including; the individual tested organism, NR stock preparation, measurement in different days and cell density in the sample.

Finally, it is recommended the following conditions for in vivo quantification of neutral lipids using NR stain; 20% DMSO (v/v), NR concentration of $2 \mu\text{g ml}^{-1}$, an incubation time of 20 min and to be incubated at room temperature (20 °C), these are the optimal conditions for measuring the fluorescence intensity using the green alga *N. salina*.

Confocal microscopy study of lipid content in algal cells

TAGs are the key material for biodiesel production; the oil content in different algal species is variable as in some species it ranged between 50 and 60% of their dry weight (Abou-Shanab et al. 2011; Wirshing and Minocha 2012). It is clear that, changing temperature either increasing or decreasing was found to affect the total lipid productivity of microalgae. Nutrient stress including nitrogen, phosphorus starvation, light irradiation, pH, temperature, and

heavy metals was also found to affect lipid productivity (Miao and Wu 2006; Shuo et al. 2012).

It was apparent that, neutral lipid accumulation of *Chlamydomonas reinhardtii* at elevated temperature is higher compared to non-stressed *Chlamydomonas* wild type (Yao et al. 2012). Also, a remarkable increase of neutral lipid accumulation in *Isochrysis galbana* and *Ochromonas danica* was observed as a result of increasing temperature from 15 to 30 °C. However, shifting the temperature from 25 to 12 °C increased the production of unsaturated lipids by 20% in *Dunaliella salina* (Sharma et al. 2012).

To identify algae species with high lipid content, lipophilic fluorescent dye Nile red which is commonly used as a vital stain for lipid-containing vesicles [also referred to as lipid bodies (LB)] was used within algal cells (Bertozzini et al. 2011; Chen et al. 2009; Cirulis et al. 2012; Cooper et al. 2010; Eibl et al. 2014; Greenspan and Fowler 1985; Siaut et al. 2011; Wang et al. 2009). For the microscopy study, cells were grown to stationary phase. After harvesting, aliquots of Nile red stock solution in 20% DMSO were added directly to the algal suspensions, prior to the microscopic study as a pretreatment to increase efficiency of staining (Chen et al. 2009). The dye diffuses through the cell membranes and accumulates in the intracellular lipid-based compartments within several minutes. The mechanism of accumulation is called a diffusion-trap mechanism that is based on the solvation of a hydrophobic Nile red by neutral lipids (Akimoto and Mimuro 2007).

Labeling efficiency of Nile red differs for various algal species due to the variation of their cell-wall thicknesses (Cabanelas et al. 2015). For this reason, algal cells were gently stirred over a time period of 15 s after addition of Nile red to accelerate the diffusion of dye into the cells. Importantly, no vital staining with Nile Red or stirring affected the cell viability in our study.

The results of staining experiments of algal cells with Nile red are shown in (Fig. 2). Individual lipid bodies are clearly distinguishable; LBs (green) are located in the cytoplasm and separated from the large chloroplasts (red), which exhibit moderate fluorescence from the endogenous chlorophyll. Importantly, the microscopic study allows for evaluation of the LB sizes; thus the corresponding lipid quantity can be estimated for each type of algae (Table 1).

Figure 2 shows that all lipid vesicles have a spherical shape and range in size from 0.2 to 1.5 μm depending on the strain. In some cases Nile red can stain cytoplasmic compartments other than lipid bodies (Cabanelas et al. 2015), it can bind to certain proteins that contain a hydrophobic domain in the cytoplasm or to phospholipids, thus interfering with the LB stain and contributing to a background signal (Brown et al. 1992). This could explain the observed weak fluorescent signal (green) originating

Fig. 2 Visualization of lipid vesicles under temperature stress. Confocal fluorescence microscopy study of lipid content in different algal species: **a**—*Micractinium* sp. YACCYB33, **b**—*Synechocystis* sp. PAK12, **c**—*Pedinomonas noctilucae* C34, **d**—*Chlorella sorokiniana* KLL-G018, **e**—*Synechocystis* sp. ElfSCS31, **f**—*Chlorella variabilis* NC64A, **g**—*Synechocystis* sp. PCC 6803, **h**—*Desmodesmus pannonicus* GM4n, **i**—*Desmodesmus intermedius* CCAP 258/36. Lipophilic fluorescent dye Nile red is used as a vital stain for visualization of the oil-containing lipid bodies. The overlay shows auto-fluorescence of chlorophyll in chloroplasts (red) and Nile red stained lipid bodies (green) within algal cells. In some cases, lipid bodies appear yellow due to their spatial overlap with chloroplasts. Excitation of Nile Red was at 488 nm, emission was collected using a 530–600 nm band-pass filter. Excitation of chlorophyll fluorescence was at 633 nm, emission was imaged through a 650 nm long-pass filter

Table 1 Evaluation of lipid vesicles size (μm), volume (μm^3) and lipid content (g)/cell for the tested microalgae species based on confocal microscopy study

Microalgal species	Size (μm)/cell	Volume (μm^3)/cell	Lipid content (g)/cell at 40 °C
<i>Micractinium</i> sp. YACCYB33	0.2–0.9	0.02–1.53	1.59E–12
<i>Synechocystis</i> sp. PAK12	0.3–0.5	0.06–0.26	3.88E–12
<i>Pedinomonas noctilucae</i> C34	0.4–0.9	0.13–1.53	2.87E–13
<i>Synechocystis</i> sp. PAK13	0.2–0.5	0.02–0.26	8.19E–13
<i>Chlorella sorokiniana</i> KLL-G018	0.2–1.1	0.02–2.79	8.26E–13
<i>Synechocystis</i> sp. ElfSCS31	0.6–0.8	0.45–1.07	5.75E–13
<i>Chlorella vulgaris</i> BDUG 30061	1.1–1.3	2.79–4.60	4.30E–13
<i>Cyanobacterium aponinum</i> ThrSCCsp4	0.2–1.3	0.02–4.60	4.91E–13
<i>Chlorella variabilis</i> NC64A	0.2–0.5	0.02–0.26	6.81E–13
<i>Synechocystis</i> sp. PCC 6803	0.4–1.2	0.13–3.62	1.26E–12
<i>Desmodesmus pannonicus</i> GM4n	0.3–0.9	0.06–1.53	6.33E–13
<i>Desmodesmus intermedius</i> CCAP 258/36	0.3–1.5	0.03–7.07	1.01E–12

from the cellular membrane in samples *Micractinium* sp. YACCYB33 and *Pedinomonas noctilucae* C34 (Fig. 2a, c).

TAGs enhancement and accumulation as a result of physiological and environmental stress has been reported by many researchers. In this study, we investigated the

accumulation of lipid vesicles as a direct effect of temperature stress (40 °C). NR was found to be a powerful fluorescence dye that can visualize and quantify lipid vesicles with the use of laser microscope after pretreatment with DMSO as an effective carrier for NR across the rigid

cell wall to the interior of the microalgal cell and stain it in situ.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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